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Primary cells from the anlage of the rat suprachiasmatic nucleus (SCN) have been immortalized by infection with a retroviral vector encoding the adenovirus E1A oncogene. The resulting neural cell lines (SCN1.4 and 2.2) are characterized by extended growth potential without neoplastic activity, uniform nuclear expression of E1A protein and heterogeneous cell types in various stages of differentiation. The SCN1.4 and SCN2.2 lines exhibit many cells with glial morphologies and a small, stable population of cells with neuronal characteristics. Differentiated neuron-like cells are distinguished by fine processes and immunostaining for neuronal markers and peptides found within SCN neurons *in situ*. Concordant with immunostaining data, content, release and mRNA expression of SCN neuropeptides in both lines followed a distinct pattern with somatostatin and vasopressin cells representing the most and least common peptidergic phenotypes, respectively. Since E1A-immortalized cells from the primordial SCN can differentiate into neurons with mature, parental-like phenotypes, the initial project objective was to determine whether the lines also retain the distinctive function of the SCN to generate circadian rhythms. Circadian wheel-running activity was restored in ≈40% of SCN-lesioned hamsters following transplantation of immortalized cells, suggesting that circadian timekeeping may be a stable functional property of these lines. The project has also yielded clonal lines of immortalized cells that exhibit specific SCN phenotypes and may provide models for studying the regulation of neuropeptide gene expression and the role of peptidergic cells in mammalian circadian timekeeping.

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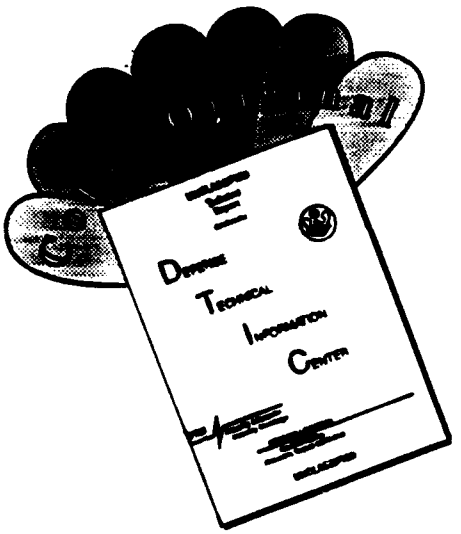

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INTRODUCTION

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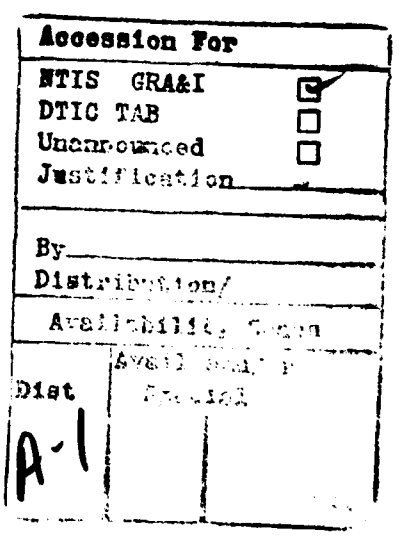
In mammals, the internal biological clock responsible for the generation of circadian rhythms in a variety of biochemical, physiological and behavioral activities is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Moore, 1983; Turek, 1985). Evidence for the integral role of the SCN in the central neural control of mammalian circadian rhythms has been established over the past two decades through the application of ablation, transplantation and *in vitro* isolation techniques to study its pacemaker function (Rusak and Zucker, 1979; Earnest, and Sladek, 1987; Gillette and Reppert, 1987; Ralph and Lehman, 1991). Complementary to this analysis of SCN circadian function, anatomical studies have yielded considerable insight into the complex neural organization of the SCN and the variety of histochemically-distinct neurons found within this region (Card and Moore, 1984; van den Pol and Tsujimoto, 1985; Moore and Speh, 1993). However, many key questions concerning the relationship between the circadian function and specific cellular components of the SCN remain unanswered. For example, it is unknown as to what cell type(s) within the SCN are critical for its function in the generation of circadian signals and whether pacemaker function is an endogenous property of individual cells or depends on the interaction of an orchestrated group of cells within the SCN.

Further progress in elucidating the cellular nature of the SCN circadian pacemaker may require the application of novel experimental strategies because fundamental properties of the SCN and its constituent neurons often compromise the utility of classical techniques to address pertinent issues. In particular, the diversity and mortal status of neurons within the SCN present formidable obstacles in the use of primary cell cultures to selectively isolate large populations of specific cell types and analyze their capacity to generate circadian rhythms. Organotypic and dissociated cell cultures have been developed to isolate SCN neurons and study their composite pattern of endogenous cellular activity *in vitro* (Earnest, and Sladek, 1987; Gillette, and Reppert, 1987; Bos and Mirmiran, 1990; Wray et al., 1993), but these preparations require cultivation of all or large subregions of the SCN and thus provide little resolve to the problematic issue of SCN heterogeneity in establishing pure neuronal populations.

Moreover, the post-mitotic nature of neuronal elements and variability in their survival *in vitro* impose limitations on both the number of neurons with a common genetic background that can be cultivated for primary SCN cultures and the uniformity of these preparations with regard to their cellular composition.

Development of mitotic, immortal cell lines derived from progenitors could provide a viable strategy to circumvent some problems that features of the SCN impose in establishing large, pure populations of specific SCN cell types. In attempt to institute this strategy, we utilized gene transfer techniques to mediate the introduction of an oncogene and expression of its immortalizing protein product in primary SCN cells because this method has yielded many useful cell lines that express stable growth characteristics and retain parental phenotypic properties (Cepko, 1988, 1989, 1990). Since the 12S protein product of the adenoviral early-region 1A (E1A) gene has been shown to induce DNA synthesis and extend the growth potential of primary cells derived from a variety of rat tissues (Quinlan and Grodzicker, 1987; Cone *et al.*, 1988; Ryder et al., 1990), the capacity of this oncogene to immortalize progenitors of the rat SCN was explored in the present experiments. The results reported here indicate that transfer of the 12S E1A gene into primary SCN cells in culture using retroviral vector constructed by Cone and coworkers (1988) yields growth-stimulated cell lines that express glial as well as neuronal phenotypes. In addition, characterization of these lines after drug selection suggests that SCN-like antigenic properties, such as the expression of neuropeptides, and circadian timekeeping function are retained by immortalized cells.



EXPERIMENTAL PROCEDURES

Animals and Primary Cultures

On separate occasions at day 15 and 16 of gestation, fetuses were removed from timed-pregnant Long-Evans dams (N=4; Charles Rivers Laboratories) and placed in an ice-cold chamber moistened with CMF buffer. Each fetal brain was removed and sectioned in the coronal plane through the anterior third of the developing hypothalamus. As established previously (Earnest et al., 1989), the fetal SCN was selectively dissected from this section using topographical landmarks described by Altman and Bayer (1978a,b) to delineate the SCN from adjacent regions of the developing hypothalamus that contain the anlagen of the paraventricular and supraoptic nucleus. Briefly, selective isolation of the presumptive anlagen of the SCN was accomplished by excising a small piece of tissue from each side of midline immediately dorsal to the optic chiasm. Tissue pieces from 12-18 fetuses were pooled and maintained in cold CMF buffer. After dissociation of the tissue by trituration in 0.15% trypsin and CMF buffer, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2μg/ml glucose, 292μg/ml L-glutamine, 2.5μg/ml fungizone and 100U/ml penicillin and 100μg/ml streptomycin. The cell suspension was plated onto poly-D-lysine- or mouse laminin-coated, 24-well plates (16mm diameter) at a density of 5 x 10^5 cells/well and incubated at 37°C.

Retrovirus Vector and Infection Protocol

Defective, recombinant retroviruses were generated by Φ2 cells (kindly provided by Dr. R.D. Cone, Cold Spring Harbor, NY) that had been transfected with retroviral vector (DOL$^-$) carrying the adenovirus 2-adenovirus 5 hybrid E1A 12S sequence and the neomycin phosphotransferase gene (*neo*) (Cone et al., 1988). Viral stock was established by collecting and filtering (0.45μm pore) 24-hr conditioned medium from confluent cultures of Φ2 cells (DMEM + 10% FCS). Retroviral infection of primary SCN cells was performed after their attachment by incubation for 1 hr at 37°C with this conditioned medium (virus titer ≈ 10^4-10^5 CFU/ml) in the presence of 2μg/ml polybrene (Aldrich).

After a second incubation with fresh virus and polybrene, cells were maintained in regular growth medium (DMEM + 10% FCS and supplements), propagated for 7-10 d, trypsinized and replated onto 35-mm dishes for expansion. Cultures were then treated with medium containing genetecin (G418; GIBCO; 250μg/ml) to select for virus-infected cells in which the vector had been integrated into their genome. G418 selection was continued for 14 d with media changes occurring every 2-3 d and surviving, drug-resistant cells were expanded by passage onto 60-mm plates. At confluence, each culture was then split 1:4 for 10-15 passages and at each stage of expansion several colonies (≈ 6 x 10^5 cells/colony) were individually frozen in cryoprotectant medium containing DMEM + 20% FCS + 7% DMSO and stored in liquid nitrogen. Throughout this propagation phase, living cultures were examined periodically using phase contrast microscopy to assess morphological characteristics of growing cells. Infection of primary SCN cells obtained on separate occasions at day 15 and 16 of gestation and parallel application of drug-selection and propagation procedures have ultimately yielded two permanent cell lines (named SCN1.4 and SCN2.2, respectively).

Cell Growth Assays

Growth curves for individual cell lines were established by plating a series of cultures at three different cell concentrations (i.e., 10^5 cells/ml, 3 x 10^4 cells/ml and 10^4 cells/ml) and calculating cell counts at 24-hour intervals for 2-3 days. The population doubling time was derived by plotting cell concentration against time. To establish a mitotic index for each cell line, approximately 5 x 10^4 cells were plated on glass coverslips, allowed to attach and then incubated for 12-18 hours in the presence of 0.5μCi/ml ^{3}H-thymidine (2 Ci/mmol). Following fixation of cells in 4% formalin (ph=7.0), coverslips were coated with Kodak NTB-2 emulsion (1:1 dilution), stored dessicated at 4°C for 3 days and developed in D-19 developer and rapid fix (Kodak). Mitotic index was calculated by counting radiolabeled cells on each coverslip as a percentage of total cell number.

Immortalized lines were also assessed for evidence of anchorage-independent growth by plating 5 x 10^3 cells in a soft agar (0.42% Noble agar in DMEM with 10% FBS) overlay on a hard agar base (0.64% agar in DMEM). Cultures were maintained at 37°C, treated with 2ml of fresh soft agar every

4 days and evaluated microscopically for colony formation after 3-4 weeks. Parallel analysis of transformed chick neuroretinal cells (LA29NR; Seigel and Notter, 1992) was conducted as a positive control for proliferation in soft agar.

Immunological Characterization

Immunocytochemistry for cell-specific antigens and neuropeptides. For immunocytochemical analysis, cells were grown on multi-well, plastic dishes or chambered, glass sides (Lab-Tek). Immunostaining with antibodies for SCN peptides and cell type-specific neuronal and glial antigens was conducted on stable colonies fixed for 5 minutes in 5% acrolein in 0.1M phosphate buffered saline (PBS). To eliminate endogenous perioxidase activity, cultures were successively treated with sodium periodate and sodium borohydride in PBS. With intermittent rinses, colonies were sequentially incubated in 5% normal donkey serum in PBS with 0.4% Triton-X and 0.25% bovine serum albumin (BSA) for 30 minutes, primary antibody in PBS with 0.25% BSA for 48-72 hr at 5°C, biotinylated donkey anti-rabbit immunoglobulin (IgG; Jackson Labs) diluted 1:1250 for 90 min at 21°C, an avidin-biotin-peroxidase complex (Elite-ABC kit; Vector Labs) for 90 min at 21°C and reacted for 5-10 minutes with a chromagen solution consisting of 0.03% DAB and 0.03% hydrogen perioxide in Tris buffer.

A mouse monoclonal antibodies against microglial cell surface proteins (MRC OX-42; Serotec) and microtubule associated protein-2 (MAP-2; Boeringer Manheim) and rabbit polyclonal antisera against S-100, glial fibrillary acidic protein (GFAP; Dako Corp.), neuron-specific enolase (NSE), protein gene product 9.5 (PGP; Ultraclone Limited), were used (at dilutions of 1:100, 1:200, 1:500, 1:2000, 1:1:7500 and 1:7500, respectively) to identify glial- and neuronal-specific antigens in immortalized cells. Expression of SCN peptides in colonies was examined using rabbit polyclonal antisera against arginine vasopressin (AVP; Arnel Products), gastrin releasing peptide (GRP), somatostatin (SMT), and vasoactive intestinal polypeptide (VIP; Peninsula Laboratories) at dilutions of 1:15000, 1:5000, 1:6000, and 1:6000, respectively. The specificity of immunostaining for SCN peptides (i.e., AVP, GRP, SMT and VIP) was verified by using standard procedural and

preabsorption controls (10^{-4}M of each peptide). Cultures were also screened with a rabbit polyclonal antibody against oxytocin (at 1:2000; Ferring) to determine whether the immortalized lines display a peptidergic immunophenotype that is indigenous to many hypothalamic cells located near the developing SCN.

Radioimmunoassay for cellular content and release of neuropeptides. After trypsinization of confluent cultures on 60-mm plates and approximation of cell number, cells were homogenized in 0.5ml of 0.1N HCl, incubated at 90°C for 90 sec to inactivate peptidases. The supernatant was removed from homogenate samples following centrifugation and stored at -70°C. In addition, culture medium was periodically collected during media changes to examine cellular release of neuropeptides.

AVP, SMT and VIP concentration in homogenate and media samples were determined by radioimmunoassay using a rabbit antisera against these peptides (Arnel Products; Peninsula Labs). The assay for AVP was performed as described previously (Earnest et al., 1991) and has a minimum sensitivity of 0.25 pg/tube and less than 0.001% cross-reactivity with oxytocin. Radioimmunoassays for SMT and VIP were performed using similar protocols, except that antibody-bound and free peptide were separated via ethanol precipitation. These assays have a minimum sensitivity of 5 and 1 pg/tube, respectively and show no significant cross-reactivity with other peptides.

Immunofluorescent staining for E1A protein. Confluent cultures grown on chambered, glass sides were rinsed with PBS and fixed for 5 min with methanol. With intermittent rinses, cultures were sequentially incubated in 10% normal goat serum in PBS with 0.5% Triton-X and 0.25% BSA for 30 min, a monoclonal antibody for the E1A gene product (M73; Oncogene Science) at 30 µg/ml in PBS with 0.25% BSA for 1 h and fluorescein-conjugated, goat anti-mouse IgG (Jackson Labs) diluted 1:10 for 2.5 hr. The slides were washed with PBS followed by water and coverslipped using DPX mountant.

Western blot analysis for E1A protein. Cells were grown to confluence on 60-mm plates, removed from culture via trypsinization, washed in PBS, suspended in hot lysis buffer (50mM Tris, pH 6.8, 2% SDS, 10% glycerol), homogenized and boiled at 90°C for 10min. Nucleic acids and debris were pelleted by centrifugation and the supernant was recovered and stored at -70°C.

Following determination of protein content by the bicinchoninic acid method (Pierce Biochemicals), aliquots (25μg) of the cell lysates containing dithiothreitol (final conc. = 100 mM) and bromophenol blue were electrophoresed on 10% polyacrylamide gels (100V for 4-5hr). Proteins were electroblotted onto nitrocellulose (Amersham) and the blot was probed with an anti-E1A monoclonal antibody (M73; Oncogene Science) at a dilution of 1:100, rinsed extensively in TBS-Tween (25 mM Tris-HCl, 0.2 M NaCl, 0.1% Tween 20), and incubated with perioxidase-linked sheep anti-mouse IgG at a dilution of 1:300. Signal was generated onto autoradiographic film by ECL enhanced chemiluminescence (Amersham).

RNase Protection Assays for Peptide mRNAs

Using the GlassMAX RNA microisolation system (GIBCO BRL), total RNA was extracted from cultures grown to confluence on 60-mm plates. Cells from 2 individual colonies were pooled together and homogenized in 1ml of a guanidinuim isothiocynanate/2-mercaptoethanol solution. After ethanol precipitation and centrifugation, the RNA pellet was suspended in a binding solution, separated from residual impurities by selective elution through a spin cartridge membrane and reconstituted in water. Prior to solution hybridization / RNase protection analysis, RNA samples were treated with DNase I to digest genomic DNA and stored at -70°C.

Using the methods of Sherman and Watson (1988), solution hybridization / RNase protection analyses were performed to determine whether immortalized cells express peptidergic mRNAs *in vitro*. Rat pre-prosomatostatin, VIP / peptide histidine isoleucine (PHI) and AVP cDNAs subcloned into pSP65, pSP64 or pGEM plasmids were kindly provided in respective order by Dr. Richard Goodman (Goodman, et al., 1983), Dr. Steven Fink (Segerson et al., 1989) and Dr. Thomas Sherman (Sherman and Watson, 1988). Radiolabeled cRNA probes of 400, 550 and 680 base pairs (specific activity ≈ 2-4 x 10^8 cpm/ug) were generated by *in vitro* transcription of linearized copies of these plasmids (Promega) using a-^{32}P-CTP (Amersham; specific activity = 800 Ci/mmol). After digestion of plasmid DNA with DNase I, transcription reactions were electrophoresed on low-melting point agarose gels (1% wt/vol) and cRNA bands of the appropriate size were excised, extracted and

resuspended in 5X hybridization buffer (0.2M PIPES, pH 6.4; 2M NaCl; 5mM EDTA). Each hybridization reaction consisted of ≈25fmol cRNA in 6µl of 5X hybridization buffer, 40% deionized formamide, yeast RNA (20µg) and total RNA extracted from ≈ 1.2 x 10^6 cells in 5ul water. The reaction was incubated at 85°C for 5 minutes and then at 50-55°C in a shaking water bath for 6-12 hours. The non-hybridized cRNA and mRNAs were digested for 60 min at 22°C with RNase A (8µg) and RNase T1 (20U) in buffer containing 10mM Tris-HCl, ph 7.5, 5mM EDTA, 0.2M NaCl and 0.1M LiCl. After terminating the RNase digestion by incubation with SDS and proteinase K, protected cRNA:mRNA fragments were recovered by phenol-chloroform extraction and fractionated on 1.3% agarose or 4% polyacrylamide gels. Radiolabeled bands were visualized from dried gels by autoradiography. Bands of the appropriate sizes removed from the gels, placed in scintillation cocktail and counted on a liquid scintillation counter for comparative analysis.

RESULTS

Mock infection with medium from uninfected Φ2 cells failed to stimulate the growth potential of primary cells derived from the developing SCN of E15 and E16 rat fetuses. In contrast, rapid cellular proliferation was induced in similar primary cultures of the SCN primordium following infection with the retroviral vector (DOL$^-$) encoding the adenovirus 2-adenovirus 5 hybrid E1A 12S gene. The enhanced growth of virus-infected cells was evident in all cultures by 1-2 days postinfection. Despite their rapid growth, virus-exposed cells maintained little contact with each other for the first several weeks postinfection; cells were either singly isolated without companions or organized into small aggregates of no more than 4-5 cells. Following viral infection, colonies were initially characterized by many small cells (≈7-10 μm in diameter) exhibiting round, phase-bright perikarya and a few large, flat cells with stellate or spindle-like morphologies. However, flat cells tended to proliferate more rapidly and became more numerous than their round counterparts during the first week of propagation.

After G418 selection for 2 weeks, mock-infected cultures of primary SCN cells exhibited widespread cytopathology with no evidence of surviving cells whereas virus-exposed cultures were typically characterized by large macroscopic colonies that contained only a few small plaques of necrotic cells. During subsequent expansion and serial passage *in vitro*, the proliferative characteristics of drug-resistant cells showed some variation over time in culture, but generally remained similar to pre-selection standards. The enhanced growth rate of immortalized cells was clearly arrested in the absence of anchorage to substrate, with detached cells exhibiting no further growth in suspension. Complete inhibition of growth was also observed upon establishment of contact between neighboring cells; at maximal densities, cells adopted a tight-packing arrangement without overgrowing the monolayer. Furthermore, the viability of these cells remained stable even when cultures were maintained at confluence for a few days. Propagation of drug-resistant colonies has ultimately yielded two permanent cell lines with separate origins from primary SCN cells obtained on either day 15 or 16 of gestation (named SCN1.4 and SCN2.2, respectively).

Immortalized colonies also retained their polymorphic nature following drug-selection. Cultures of the SCN1.4 and SCN2.2 lines were typically composed of a large population of cells (≈85-90% of colony total) that displayed a broad, flat, polygonal appearance and formed a prominent basement layer underlying widely-scattered clusters of round, phase-bright cells. Immediately after attachment, many of the round cells were further distinguished by the development of fine, bipolar or multipolar processes. Despite their differentiated profile, cells displaying a round phenotype appeared to retain the ability to divide by assimilating all processes prior to each bout of mitotic activity. In addition, most evidence of neuritic outgrowth from round cells was lost as colonies approached confluence, apparently because their processes either regressed or were masked during close cellular apposition.

Immunological methods for detection of E1A protein were applied to determine whether cell proliferation after G418 selection was associated with active expression of the immortalizing oncogene encoded by the retroviral constuct. In all G418-resistant cells surviving multiple passage, immunofluorescent staining for E1A protein was clearly evident within the cell nucleus. Immunoblot analysis of E1A protein also demonstrated that immortalized cells continued to express the E1A oncogene after numerous passages over 18 months. The levels of E1A protein detected in colonies of immortalized cells were similar to those found in comparably-sized cultures of Φ2 cells that express retrovirus encoding the E1A oncogene.

Although the present immortalization strategy employed a retrovirus vector that has been rendered defective and incapable of autonomous replication, both cell lines were evaluated for the presence of wild-type, replication-competent helper virons. Using an assay to test their ability to provide for the transfer of the neomycin phosphotransferase gene to uninfected cells, SCN1.4 and SCN2.2 cells exhibited no detectable evidence of contamination with helper virus; colonies of each cell line, when isolated in baskets with a filter floor allowing communication only via the medium, were consistently unable to confer G418-resistance upon underlying co-cultures of NIH3T3 cells. The helper-free nature of these E1A-immortalized lines was also revealed by electron microscopic analysis demonstrating that constituent cells exhibit normal ultrastructural organization and cellular organelles without any sign of free or budding virus-like particles (**Table 1**).

Proliferative characteristics of E1A-immortalized cells were specifically assessed by generating a growth curve and mitotic index for each line. After initial passages, SCN1.4 and SCN2.2 cells expressed similar growth rates and doubled in number at approximately 24-hour intervals. Consistent with their rapid rate of replication, immortalized cells exhibited high mitotic activity; nuclear incorporation of [^{3}H]-thymidine was observed within 88-92% of the cells in individual cultures of each line. Importantly, the SCN1.4 and SCN2.2 cell lines have been continuously cultured for periods ranging from several months to over a year and subjected to numerous passages (i.e., up to 60 times) without expressing any signs of crisis or senescence in their proliferative characteristics.

Despite their stimulated growth potential, SCN1.4 and SCN2.2 cells showed no evidence of transformed phenotype in several assays for tumorigenicity (**Table 1**). The growth of E1A-immortalized cells was completely arrested *in vitro* following their suspension in soft agar whereas tumorigenic control cells (LA29NR; transformed chick neuroretinal cells) exhibited continuous growth and colony formation in this substrate. The proliferative behavior of immortalized cells was also supressed *in vivo* after transplantation into the rat brain; no neoplastic activity was apparent at the graft site after four months.

Since morphological criteria were often equivocal in distinguishing the presence of glial and neuronal components in the SCN1.4 and SCN2.2 lines, the phenotypes of immortalized cells were further defined via immunocytochemical screening with cell type-specific antibodies (**Table 1**). Cultures of each cell line were examined at various stages of growth and differentiation for expression of the glial cell markers, OX-42, GFAP and S100 and the neuron-specific antigens, PGP, NSE and MAP-2. During initial passages, a few (≈1-5%) of the large cells with flat morphologies were observed to express the astrocytic filament protein, GFAP, in a diffuse pattern within the cell cytoplasm. However, cultures were characterized by the complete loss of this GFAP expression after 5-10 passages of the cells. Furthermore, no GFAP expression was detected in subsequent colonies irrespective of whether analysis was conducted during their growth phase or at confluence. Even when grown in serum-deprived medium (N2; Bottenstein and Sato, 1979) or treated with succinylated concanavalin A or agents that elevate cAMP to enhance differentiation, cultures were continuously

marked by the absence of GFAP-positive cells. At all stages of passage, growth and differentiation, the SCN1.4 and SCN2.2 lines were characterized by a number of large, polygonal cells with cytoplasmic immunoreactivity for the microglial marker, OX-42, but were devoid of cells expressing the astroglial calcium-binding protein, S100. In contrast, NSE, PGP and MAP-2 were expressed by both of the immortalized lines in a uniform and stable pattern over time. Immunostaining for each of these neuron-specific antigens was clearly evident in all colonies and exclusively displayed within the perikarya of small cells exhibiting round morphologies. In the presence of serum-supplemented medium, approximately 20-40% of the round cells displayed cytoplasmic immunoreactivity for NSE, PGP or MAP-2. The proportion of round cells expressing these neuronal markers increased (up to ≈80%) after several days of growth in N2 medium. Under all conditions tested, no cells with flat morphologies were ever observed to express NSE, PGP or MAP-2.

Further immunocytochemical analysis was performed to determine whether the cell lines retain SCN-like antigenic characteristics. Immortalized cells were specifically examined for expression of arginine vasopressin (AVP), gastrin releasing peptide (GRP), somatostatin (SMT) and vasoactive intestinal polypeptide (VIP) because these, and many other, peptides are found within distinct neuronal populations in the SCN *in vivo* (Moore, 1983; Card and Moore, 1984; van den Pol and Tsujimoto, 1985). Both growing and confluent colonies of the SCN1.4 and SCN2.2 lines exhibited a distinct population of cells exhibiting cytoplasmic immunoreactivity for AVP, GRP, SMT or VIP. Similar to the pattern of immunostaining for neuron-specific antigens, expression of SCN neuropeptides was evident in small, round cells (**Fig. 1**), but not in any cells displaying a flat appearance. Cells expressing AVP, GRP, SMT or VIP were usually organized into small, scattered clusters and characterized by localization of immunoreactivity within the cell body. The immunoreactive profiles of many peptidergic cells, especially those containing SMT or VIP, were distinguished by a more differentiated appearance when colonies were grown on laminin-coated plates. In these colonies, cells expressing SMT or VIP often displayed 2-4 long immunopositive neurites (100-150μm) that projected to other cells of the same phenotype. AVP-, GRP-, SMT-, and VIP-immunoreactive cells were found in stable, but unequal, proportions across parallel cultures. In

both lines, this differential expression of peptidergic cell types tended to follow a general pattern in which SMT>VIP>GRP>AVP. Expression of all SCN peptidergic cell types was enhanced in cultures grown in N2 medium or treated with succinylated concanavalin A, such that the number of cells displaying immunoreactivity for a given peptide increased 2-5 fold relative to control colonies. The SCN-like nature of immortalized cells with peptidergic immunophenotypes was further specified by analysis indicating that the immortalized lines were devoid of oxytocin-expressing cells which are normally found in paraventricular and supraoptic regions of the hypothalamus.

To corroborate immunocytochemical findings, cells from the SCN1.4 and SCN2.2 lines were also examined for content and release of SCN neuropeptides and expression of their mRNAs. Based on radioimmunoassay for AVP, SMT and VIP in cellular homogenates and culture medium, both lines were determined to express cells that contain and secrete these peptides in compatible amounts. Determinations from individual colonies appeared to reflect the expected relationship between storage and daily output of each peptide because content was considerably greater than observed levels of release. Furthermore, solution hybridization/RNase protection analyses revealed that mRNAs for AVP, SMT and VIP were expressed at low levels in the cell lines. Consistent with immunocytochemical observations on the expression of peptidergic cell types, the levels of peptide content and release and mRNA expression generally conformed to a differential pattern such that SMT>VIP>AVP in both lines.

Since immortalized cells express SCN-like antigenic properties, further screening of the lines was conducted to determine whether these genetically-engineered progenitors of the SCN retain the circadian timekeeping function of their *in vivo* counterparts. Experiments utilized the neural transplantation technique to assess the ability of cell grafts derived from early passages of these immortalized lines to restore the circadian rhythm of wheel-running activity in arrhythmic SCN-lesioned hamsters. In these experiments, small colonies were suspended in medium and injected using a Hamilton syringe into the vicinity of the ablation site in SCN-lesioned hamsters that had exhibited a loss of circadian rhythmicity in their wheel-running activity for at least 6 weeks.

Grafts containing viable cells were histologically identified in all animals. The transplanted cells were generally organized into a single aggregation located in the host third ventricle-optic chiasm region. Grafts were usually associated with a number of macrophages near the graft-host brain interface, suggesting that transplantation of the E1A-immortalized cells evoked responses in the host immune system. Immunocytochemical analysis revealed that some grafts contained a few cells and fibers expressing neuropeptides normally found in the SCN *in situ* (i.e., VIP, GRP and AVP; **Fig. 2**). These neuropeptidergic cells were usually arranged in small clusters within the grafts and were characterized by small, ovoid perikarya.

Functional correlates of the grafted cells in restoring circadian rhythmicity in the pattern of hamster wheel-running behavior were strictly correlated with graft viability and the expression of SCN-like peptidergic neurons. Restoration of the circadian activity pattern was evident within 1-4 days after transplantation in all animals (N=4) that were found to exhibit viable grafts containing VIP, GRP and/or AVP cells and/or fibers (**Fig. 3** and **4**). In contrast, the pattern of wheel-running activity remained arrhythmic during the post-transplantation period in all SCN-lesioned hamsters with grafts that were devoid of SCN-like neuronal elements or were characterized by low survival of grafted cells due to macrophage proliferation near the graft-host interface. Future studies will continue to study hamster wheel-running activity as an index of graft function in restoring circadian rhythmicity, but hamsters with the τ mutation will be used as transplant recipients because the role of grafted cells versus host SCN cells (surviving the lesions) in determining the period of the restored rhythms can be easily distinguished.

Since the morphological and antigenic characteristics of immortalized cells indicate that the lines contain heterogeneous cell types, efforts have been initiated to clone and select for pure strains of SCN cells that exhibit specific phenotypes. Clonal lines of SCN progenitors were established using the traditional method of dilution plating in which early passages of E1A-immortalized cells were expanded and then diluted sufficiently on 96-well titer plates so that a single, viable cell formed an isolated colony. Similar to their parent cell line, most clonal populations of immortalized SCN cells exhibited rapid growth rates and expressed cellular morphologies that were more distinctive and

homogeneous than their predecessors. In fact, several clones displayed a distinct neuronal morphology characterized by small, bipolar cells with three-dimensional, rounded perikarya and thin, long processes. These clonal lines were further distinguished by immunocytochemical expression of SCN neuropeptides such as AVP and SMT (**Fig. 5**). Even after selection of a pure strain of cells, some clonal lines appeared to show considerable heterogeneity, probably due to the multipotent nature of progenitor cells. Clonal lines displaying evidence of homogeneity could provide a unique opportunity to study the circadian pacemaker mechanism in the SCN at new levels of organizatior Application of the neural transplantation technique to examine the capacity of these lines to restore circadian rhythmicity in the behavioral activities of SCN-lesioned hosts could have important implications for determining whether circadian pacemaker function is a unique property of a specific SCN cell type.

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Table 1. Properties of Cell Lines Derived from Rat SCN

	Antigenic Characterization										Other Properties	
	GFAP	S100	NSE	PGP	MAP	VIP	GRP	aVP	SS	OXY	Helper Virus	Tumor
SCN_{SMD1}	-	-	+	+	+	++	+	+	++	-	- EM - Co-cult.	- agar - graft
SCN_{SMD2}	-	-	+	+	+	++	++	+	++	-	- EM - Co-cult.	- agar - graft

FIGURE LEGENDS

Figure 1. Immunostaining for SCN neuropeptides in brightfield photomicrographs of representative colonies from the SCN 1.4 line. Colonies typically contained a number of cells expressing immunoreactivity for AVP, GRP, SMT or VIP. Immunostaining in these cells was mainly restricted to the cell body, although some immunoreactive fibers are evident in the micrographs.

Figure 2. Immunostaining for SCN neuropeptides in brightfield photomicrographs of immortalized cell grafts in the third ventricle region of a SCN-lesioned hamster (#AU). VIP- and GRP-immunopositive fibers emanate from the ventral aspect, forming a bridge between the graft and the underlying host optic chiasm. A few VIP- and GRP-immunoreactive perikarya are located just dorsal to this fiber connection between the graft and host brain. AVP-immunopositive perikarya and fibers are segregated in a different portion of the graft; these vasopressinergic elements are localized in the dorsal limit of the graft and are associated with the host paraventricular nucleus.

Figures 3 and 4. Double-plotted wheel-running activity records of two SCN-lesioned male hamsters in which grafts of immortalized SCN cells restored free-running rhythms during exposure to constant darkness. The transplant recipient in Figure 4 showed a split pattern of activity that persisted throughout the post-grafting interval. Power spectral analysis indicated that circadian periodicity was not detectable after lesioning, but was restored following grafting. Days used for spectral analysis are delinated by the brackets at the right of the activity record.

Figure 5. Representative brightfield photomicrographs illustrating the effect of treatment with the differentiating agent, succinylated concanavalin A (SCA), on somatostatin (SS) expression in immortalized cell colonies derived from a clonal line ($SCN_{4.33}$). LEFT PANEL: Untreated colony (CONTROL) containing predominantly neuron-like cells that show limited expression of SS-immunoreactivity. RIGHT PANEL: SCA-treated culture exhibiting a dramatic increase in the number of SS-immunoreactive neurons relative to control colonies.

FIGURE 1

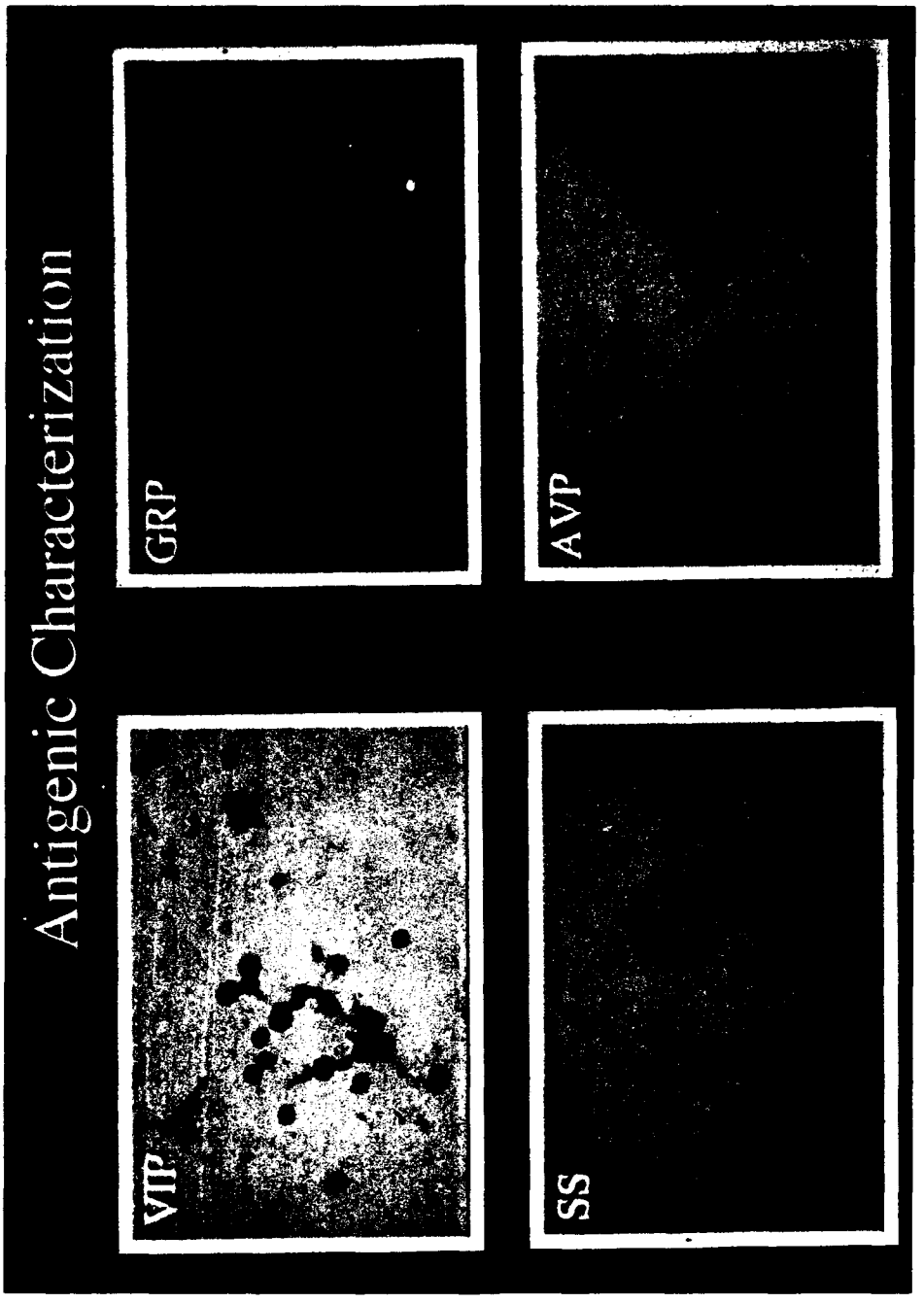

FIGURE 2

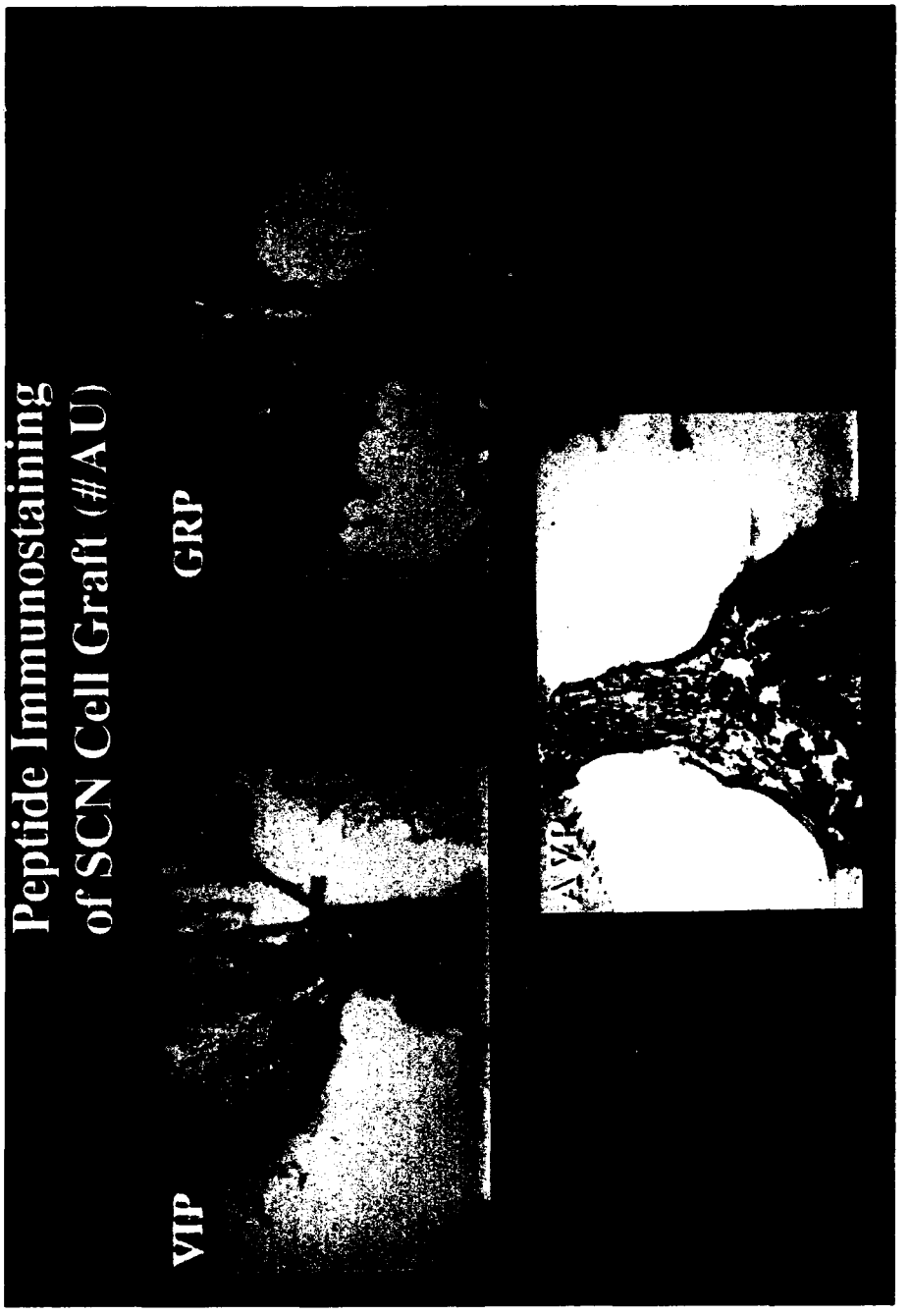

FIGURE 3

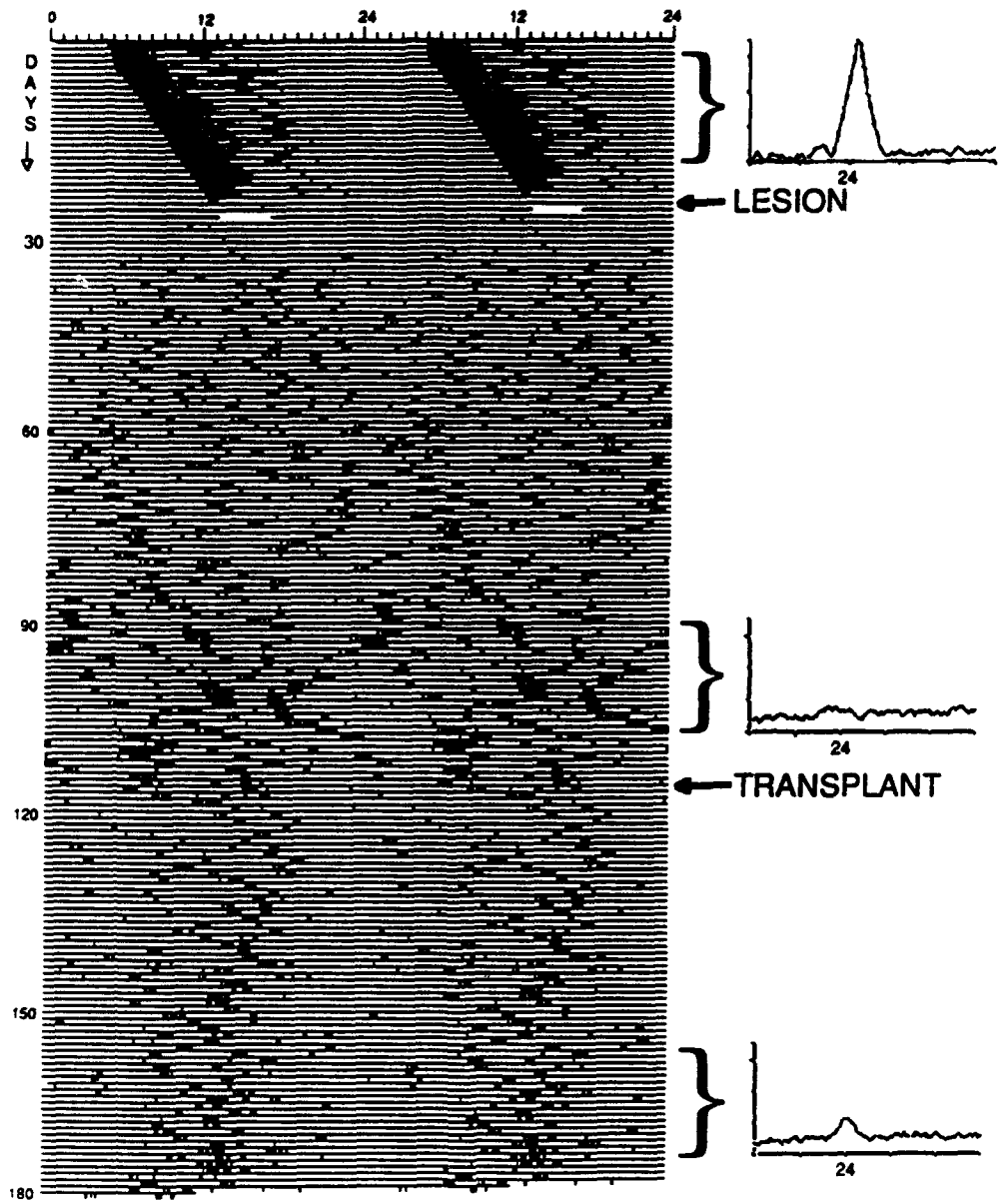
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FIGURE 4

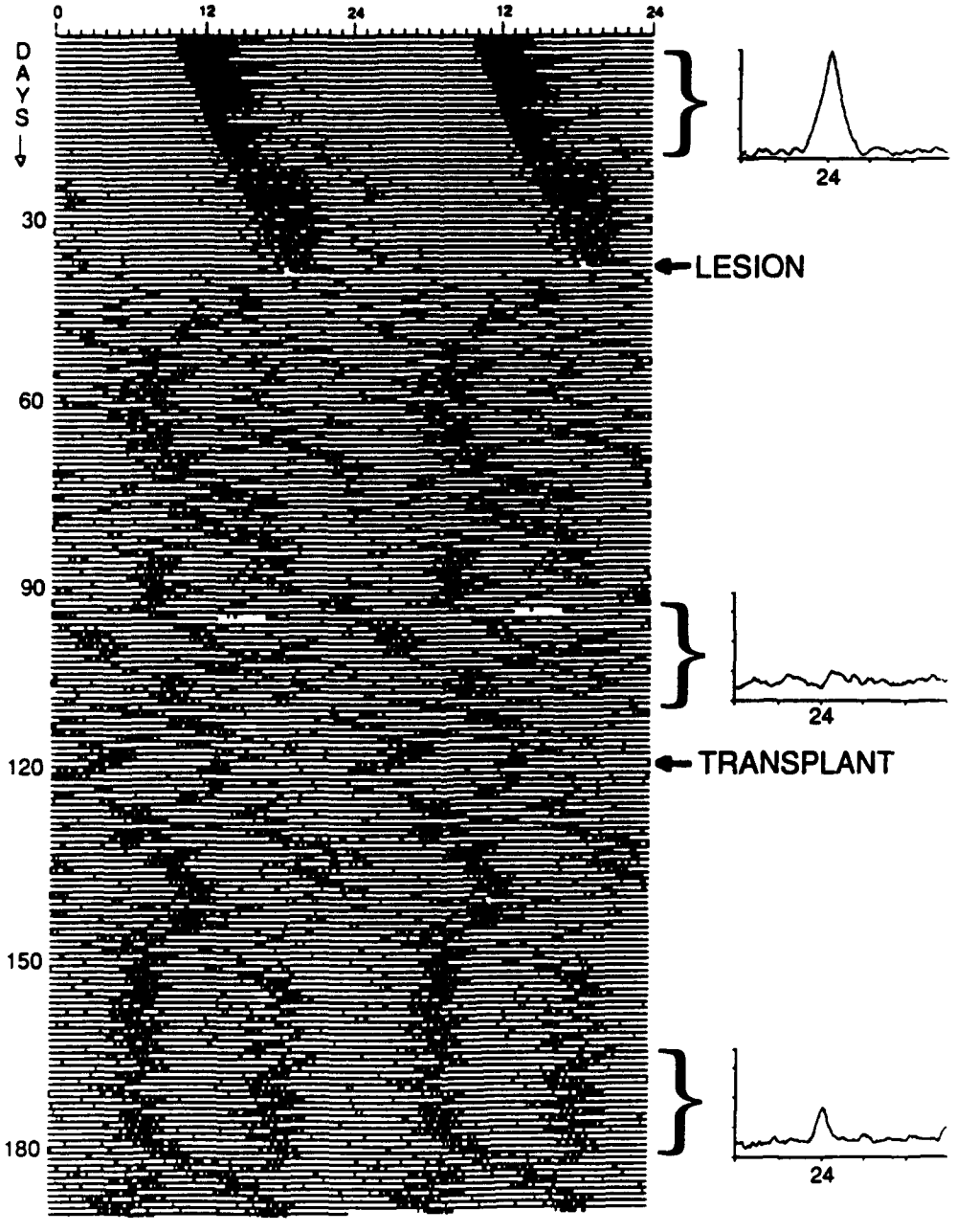
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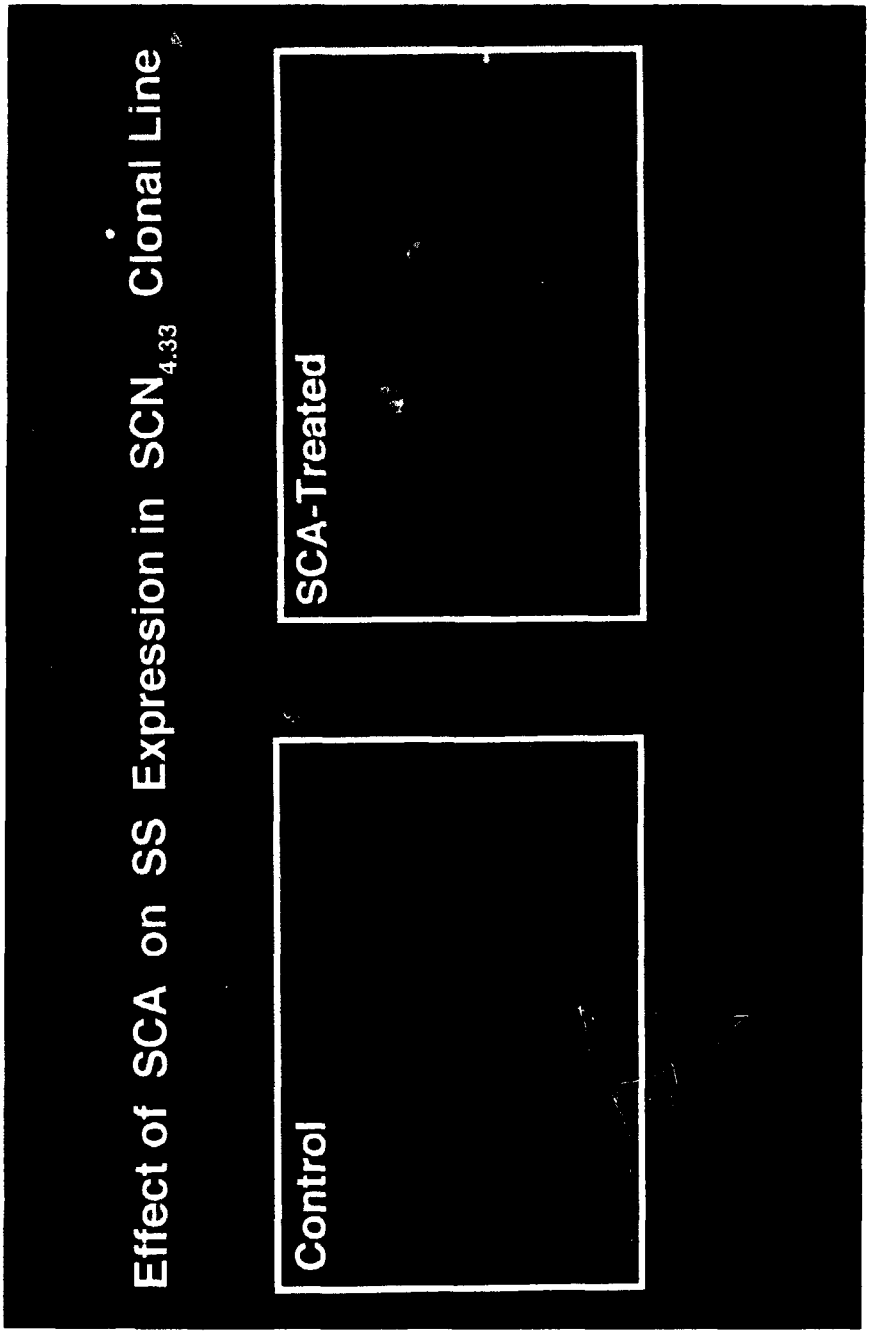
FIGURE 5


Effect of SCA on SS Expression in SCN4.33 Clonal Line
Control
SCA-Treated